Developmental Biology 346 (2010) 80–89

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

# E2F1 and E2F2 have opposite effects on radiation-induced p53-independent apoptosis in *Drosophila*

Anita Wichmann [1], Lyle Uyetake, Tin Tin Su *

*Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80209-0347, USA*

ARTICLE INFO

*Article history:*
Received for publication 16 April 2010
Revised 15 July 2010
Accepted 17 July 2010
Available online 24 July 2010

*Keywords:*
Drosophila
Radiation
Apoptosis
p53
E2F

ABSTRACT

The ability of ionizing radiation (IR) to induce apoptosis independent of p53 is crucial for successful therapy of cancers bearing p53 mutations. p53-independent apoptosis, however, remains poorly understood relative to p53-dependent apoptosis. IR induces both p53-dependent and p53-independent apoptoses in *Drosophila melanogaster*, making studies of both modes of cell death possible in a genetically tractable model. Previous studies have found that *Drosophila* E2F proteins are generally pro-death or neutral with regard to p53-dependent apoptosis. We report here that dE2F1 promotes IR-induced p53-independent apoptosis in larval imaginal discs. Using transcriptional reporters, we provide evidence that, when p53 is mutated, dE2F1 becomes necessary for the transcriptional induction of the pro-apoptotic gene *hid* after irradiation. In contrast, the second E2F homolog, dE2F2, as well as the net E2F activity, which can be depleted by mutating the common cofactor, dDp, is inhibitory for p53-independent apoptosis. We conclude that p53-dependent and p53-independent apoptoses show differential reliance on E2F activity in *Drosophila*.



## Introduction

In multicellular organisms, apoptosis is important for tissue homeostasis and for eliminating damaged or abnormal cells. Tumor suppressor p53 has a well-established role in inducing apoptosis in response to DNA damage caused by genotoxic agents such as ionizing radiation (IR). Mutations in p53 and defects in the apoptotic response are common features of human cancer cells. Loss of functional p53 can render cancer cells resistant to the therapeutic effects of IR [reviewed by Cuddihy and Bristow (2004)]. Thus, understanding p53-independent pathways that induce apoptosis may be important for treating p53-deficient tumors.

Transcription factor E2F1 is best known for its role in regulating the G1/S transition via transcriptional induction of 'S phase genes' such as *cyclin E* and *DNA polymerase-α*. Overexpression of E2F1 can also transcriptionally induce pro-apoptotic genes including *p73*, *Apaf-1* and *caspase 3* and is sufficient to induce p53-independent apoptosis both in cultured mammalian cells and *in vivo* in mouse testes (Holmberg et al., 1998; Irwin et al., 2000; Moroni et al., 2001; Nahle et al., 2002; Shu et al., 2000). It has been difficult to test, however, whether E2F1 is necessary for p53-independent apoptosis because mammalian E2F1 is just one member of a large family of proteins that exhibit functional redundancy [reviewed by DeGregori (2002); DeGregori and Johnson (2006)]. E2F1, E2F2 and E2F3a are considered transcriptional activators because they activate the transcription of E2F target genes such as *cyclin E*. E2F4 and E2F5 are referred to as repressor E2Fs because they repress transcription of E2F target genes when associated with Rb proteins [reviewed by DeGregori and Johnson (2006)]. E2F6, E2F7 and E2F8 also act as transcriptional repressors, but they function independent of Rb. E2F1 through E2F6 must dimerize with a cofactor, DP, in order to bind DNA with high affinity and regulate transcription (Bandara et al., 1993; Milton et al., 2006; Rogers et al., 1996; Wu et al., 1995; Zhang and Chellappan, 1995). Mammals have 3 DP proteins, DP1, DP2/3 and DP4; whether the different DP proteins influence E2F activity differently is not well understood. Recent studies have revealed that classification of E2Fs as either activators or repressors is overly simplistic and that most E2Fs can function as both transcriptional activators and repressors depending on cellular context (Beijersbergen et al., 1994; Ma et al., 2002; Morris et al., 2000).

Compared to the mammalian E2F family, the *Drosophila* E2F family is simpler and includes just dE2F1 and dE2F2. The *Drosophila* genome encodes only one DP cofactor, dDP, and both dE2F1 and dE2F2 must dimerize with dDP in order to bind DNA (Dynlacht et al., 1994; Frolov et al., 2001). dE2F1 is considered to act primarily as an activator while dE2F2 acts mainly as a repressor, at least in the G1/S transcription program (Dynlacht et al., 1994; Frolov et al., 2001; Ohtani and Nevins, 1994). Like mammalian E2F1, dE2F1 plays a role in apoptosis. Overexpression of dE2F1 induces the expression of pro-apoptotic

* Corresponding author. Department of Molecular, Cellular and Developmental Biology, University of Colorado, 347 UCB, Boulder, CO 80309-0347, USA. Fax: +1 303 492 7744.
*E-mail address:* Tin.su@colorado.edu (T.T. Su).
[1] Current address: Center for Cardiovascular and Metabolic Research, Sahlgrenska University Hospital, 41345 Göteborg, Sweden.

0012-1606/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2010.07.023

genes, including *Dark/hac-1* and *reaper*, and results in apoptosis (Asano et al., 1996; Du et al., 1996; Zhou and Steller, 2003). Mutations in *Drosophila ago,* a ubiquitin ligase, also result in elevated dE2F1 activity, induction of *hid* and *rpr*, and apoptosis (Nicholson et al., 2009). The role of dE2F1 in IR-induced apoptosis is more complex. dE2F1 and dDP are pro-apoptotic in most larval cells examined; mutations in dE2F1 or dDP result in decreased IR-induced apoptosis in most cells of the eye and wing imaginal discs (Moon et al., 2008, 2005). The exception is the narrow band of cells at the dorsal/ventral margin of the wing imaginal disc (so-called zone of non-proliferating cells or ZNC), where dE2F1 and dDP play an anti-apoptotic role (Moon et al., 2005). dE2F2 mutant wing discs undergo IR-induced apoptosis normally. Thus, dE2F1/dDP has a pro-apoptotic role in most larval imaginal disc cells whereas dE2F2 appears to play little or no role in the process. Since loss of dDP would resemble the loss of all E2F activities in the cell, the phenotype of dDP mutants suggests that net E2F activity is normally pro-apoptotic for most cells.

In the studies discussed in the preceding paragraph, apoptosis was assayed typically at 4 h after exposure to 40 Gy (4000 R) of IR. Under these conditions, apoptosis is p53-dependent (Brodsky et al., 2000a; Moon et al., 2008; Ollmann et al., 2000). At longer times after irradiation, null mutants of *Drosophila* p53 or its presumptive activator Chk2 kinase still undergo apoptosis (Wichmann et al., 2006). Unlike the mammalian p53 family, which consists of p53, p63 and p73, *Drosophila* has a single p53; as such *Drosophila* p53 null mutants are thought to lack all p53 activities. Genetic analysis placed Chk2 and p53 in a single linear pathway, which allows us to refer to apoptosis in each mutant as Chk2-/p53-independent apoptosis. Chk2-/p53-independent apoptosis in larval wing imaginal discs is detectable at 18 h after exposure to 4000 R of X-rays (i.e. with a 12+ h delay relative to p53-dependent apoptosis) and requires caspase activity and the pro-apoptotic Smac/DIABLO orthologs. Of the latter group of proteins, Hid appears to be the key: *hid* transcript levels increased in p53 mutants at 18 h after irradiation and mutations in *hid* severely reduced p53-independent apoptosis (McNamee and Brodsky, 2009; Wichmann et al., 2006). The level of IR-induced p53-independent apoptosis can be further increased by mutations in *grapes* (encoding Chk1) or *puckered* (encoding an inhibitor of JNK signaling) while the overexpression of *puc* reduced the level of p53-independent apoptosis (McNamee and Brodsky, 2009). Thus Chk1 and JNK signaling negatively and positively regulate the level of p53-independent apoptosis respectively, but neither seems to be essential for this mode of cell death. How these or other signaling pathways result in changes in *hid* transcription in response to IR and in the absence of p53 remains to be understood.

We report here that transcription factor dE2F1 is necessary for the induction of *hid* transcription and for apoptosis following IR exposure. The requirement for dE2F1 appears to be to counteract dE2F2; the removal of both E2F activities using mutations in dDp restores apoptosis in p53 mutants. Thus, net E2F activity appears to be anti-death for p53-independent apoptosis. This is in contrast to p53-dependent apoptosis, where the net E2F activity is mostly pro-death as discussed earlier. If conserved in mammals, the role of E2F proteins uncovered by *Drosophila* studies may have important implications for radiation therapy of cancers. For example, pharmacological inhibition of net E2F activity would synergize with radiation to kill p53 mutant tumor cells whereas it would antagonize apoptosis-induction by radiation on $p53^{+}$ tumors.

## Results

### *dE2F1 is required for Chk2-/p53-independent apoptosis*

In order to identify genes that regulate IR-induced apoptosis in the absence of Chk2 and p53, we tested candidates with known roles in DNA damage responses in different experimental systems. dE2F1 is essential for G1/S transition; *de2f1* homozygous null mutants display severe growth defects and do not progress beyond the 1st larval instar (Royzman et al., 1997). To study the role of dE2F1 in apoptosis in the third-instar larval imaginal discs, we used a combination of a partial loss-of-function allele of *de2f1*, $de2f1^{i2}$, together with a null allele, $de2f1^{7172}$. $de2f1^{i2}$ results from a premature stop codon predicted to produce a truncated protein that contains the DNA binding domain but lacks the Rb-binding or the transcriptional activation domains (Royzman et al., 1999). $de2f1^{7172}$ results from a P-element insertion 33 nucleotides upstream of the start codon; no dE2F1 protein is detectable in $de2f1^{7172}$ homozygous embryos (Asano et al., 1996; Duronio et al., 1995). We generated double mutants of $de2f1^{i2}$/$de2f1^{7172}$ with *chk2* or *p53* and analyzed apoptosis by staining with the vital dye acridine orange (AO). AO specifically stains apoptotic, but not necrotic, cells in *Drosophila* (Abrams et al., 1993).

We find that both *chk2, de2f1* and *p53, de2f1* double mutants show little apoptosis compared to *chk2* and *p53* single mutants at 18 and 24 h after exposure to 4000 R of X-rays, indicating that dE2F1 is required for Chk2-/p53-independent apoptosis (Fig. 1A, quantified in B). The amount of apoptosis seen in the double mutants at these times is similar to the level seen in un-irradiated controls (compare with Fig. 2B, "0 h" time point). *de2f1* single mutants show robust apoptosis at similar times ($de2f1^{i2}$/$de2f1^{7172}$; Fig. 2 shows the 18 h time point). These data suggest that in the presence of p53, *de2f1* status does not matter at these times after irradiation, but in the *p53* mutant background, *de2f1* becomes necessary for IR-induced apoptosis.

### *A hid>GFP transcriptional reporter is sensitive to p53 and dE2F1 status*

We have previously found that Chk2-/p53-independent apoptosis is sensitive to the dosage of pro-apoptotic genes, *hid, rpr* and *grim;* heterozygotes of a chromosomal deficiency, H99, that remove the three genes show little or no Chk2-/p53-independent apoptosis after irradiation (Wichmann et al., 2006). Mutations in *hid* have a similar effect, either in the p53 mutant background (McNamee and Brodsky, 2009), or in the Chk2 mutant background (Fig. 6A and B). Furthermore, the *hid* promoter region contains E2F consensus binding sites and dE2F1 has been shown to bind *hid* promoter by ChIP assays in imaginal discs and in S2 cells (Moon et al., 2005; Tanaka-Matakatsu et al., 2009). For these reasons, we hypothesized that the requirement for dE2F1 in p53-independent apoptosis could be explained by its role in the transcriptional regulation of *hid*. To test this idea, we used a previously characterized reporter in which GFP transcription is under the control of a 2 kb fragment of the *hid* promoter that spans +16 to −2210 with respect to transcription start (to be called "*hid*>GFP reporter"). GFP signal from this reporter increased 4 h after irradiation with 4000 R, mirroring the transcriptional induction of *hid* (Tanaka-Matakatsu et al., 2009). Thus the 2 kb enhancer includes sequences that allow transcriptional induction in response to IR. The GFP signal remains elevated 24 h after irradiation, which is consistent with elevated levels of apoptosis at similar times (Fig. 3A, 'WT'; (Wichmann et al., 2006)).

We find that IR-induced increase in *hid*>GFP expression is p53-dependent; there was no discernable increase in GFP after irradiation when the reporter was in the p53 mutant background (Fig. 3A, 'p53,' 4 h time point). At 24 h after irradiation, however, an increase in GFP was detected even in p53 mutants, which is in agreement with the occurrence of p53-independent apoptosis at such times (Wichmann et al., 2006). The GFP signal at 24 h after irradiation in p53 mutants is not as high as in wild type or heterozygous sibling controls (~60%, quantified in D). This is again in agreement with the finding that induction of apoptosis by IR in p53 mutants is also not as high as in wild type (Wichmann et al., 2006). Thus, *hid*>GFP reports not only p53-dependent *hid* induction at 4 h after irradiation, but also p53-independent induction at 24 h after irradiation.

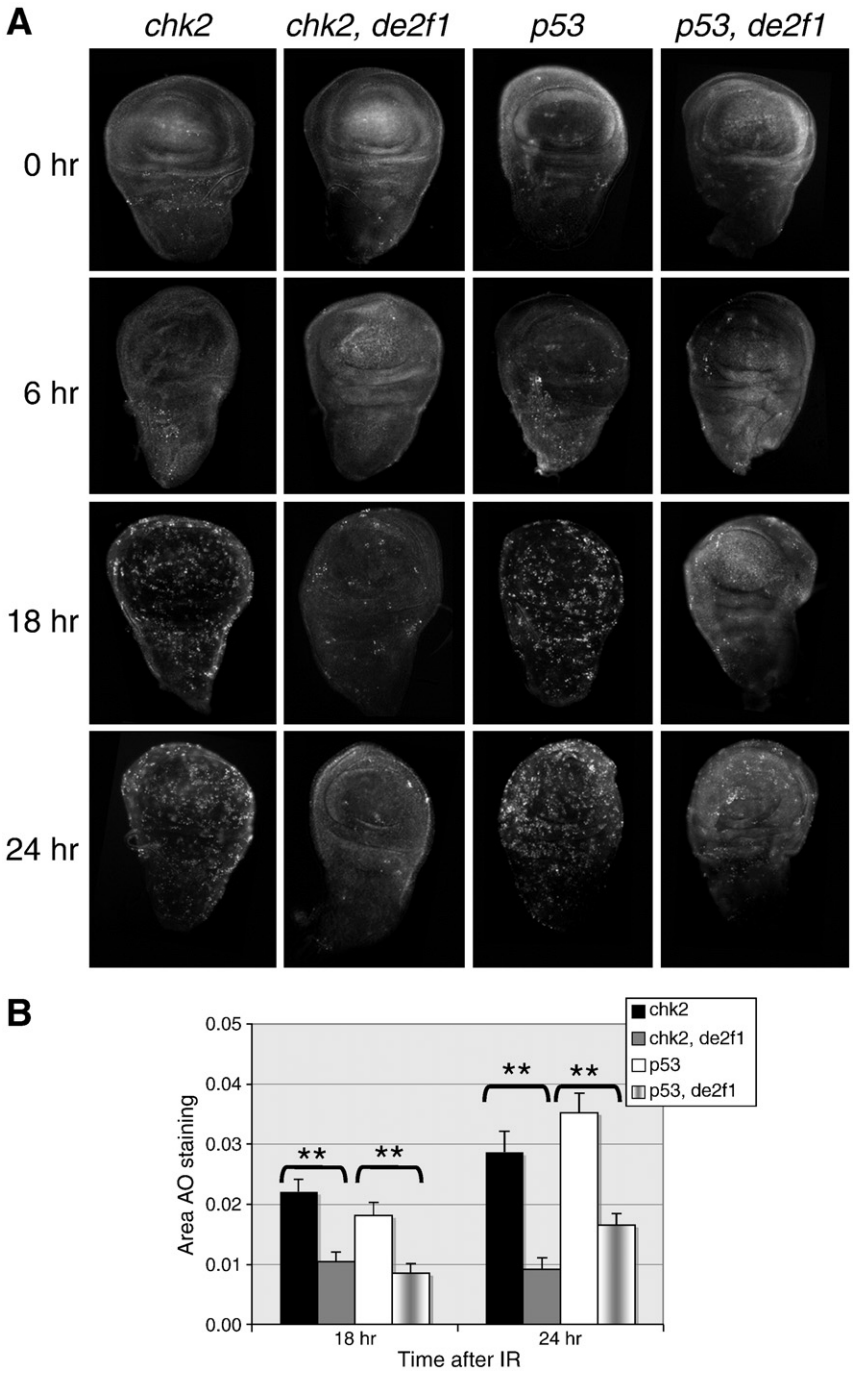


**Fig. 1.** dE2F1 is required for Chk2-/p53-independent apoptosis. (A) 96- to 125-hour-old feeding stage third-instar larvae were irradiated with 4000 R of X-rays. Imaginal discs were dissected at various times after irradiation and stained with acridine orange (AO) to detect dead cells. Genotypes are indicated for $mnk^{p6}$ (*chk2*), $mnk^{p6}$;$de2f1^{i2}/de2f1^{7172}$ (*chk2, de2f1*), $p53^{5A\text{-}1\text{-}4}$ (p53), and $p53^{5A\text{-}1\text{-}4}$, $de2f1^{i2}/p53^{5A\text{-}1\text{-}4}$, $de2f1^{7172}$ (*p53, de2f1*). (B) Quantification of apoptosis in *chk2, de2f1* and *p53, de2f1* double mutants. The fractional area of AO staining in each disc was quantified for the 18 and 24 h time points using Image J software. Error bars represent SEM. The data are from 13 to 25 discs per genotype per time point. Asterisks (**) denote a statistically significant difference with a *p*-value of less than 0.01.

We find that induction of *hid*>GFP by IR can occur in dE2F1 mutants (p53 wild type) at both 4 and 24 h after irradiation (Fig. 3B). In contrast, in p53 dE2F1 double mutants, induction of *hid*>GFP by IR becomes compromised 24 h after irradiation compared to either p53 or dE2F1 single mutants (Fig. 3C, quantified in D). We conclude that in the absence of p53, transcription induction from the *hid* promoter becomes dependent on dE2F1. Given that *hid* is required for p53-independent apoptosis, the role of dE2F1 in transcriptional induction of *hid* can explain why p53-independent apoptosis is dependent on dE2F1.

### *dE2F2 and dDP limit IR-induced apoptosis in the absence of p53*

dE2F1 must dimerize with dDP to bind DNA and activate transcription (Dynlacht et al., 1994; Sawado et al., 1998). *dDP* mutants share some phenotypes with *de2f1* mutants, such as the lack of a G1/S transcriptional program during embryogenesis and altered pattern of apoptosis in larval wing discs after IR exposure (Duronio et al., 1998; Royzman et al., 1997). Therefore, we addressed the role of dDP in p53-independent apoptosis. Because *p53, dDP* null double mutants did not survive to the third larval instar, we used a combination of a hypomorph,

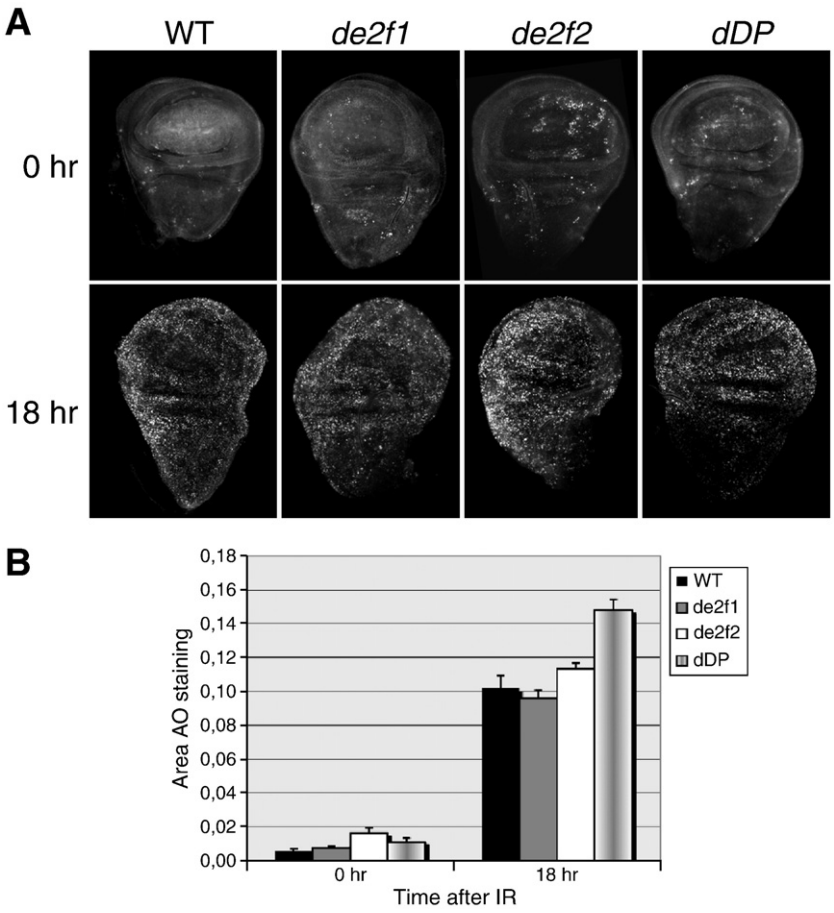


**Fig. 2.** Cell death in *de2f1*, *de2f2*, and *dDP* single mutants. (A) 96- to 126-hour-old feeding stage third-instar larvae were irradiated with 4000 R of X-rays. Imaginal discs were dissected at various times after irradiation and stained with acridine orange (AO) to detect dead cells. Genotypes are indicated for wild type (WT), $de2f1^{i2}/de2f1^{7172}$ (*de2f1*), $de2f2^{329}/de2f2^{76Q}$ (*de2f2*), and $dDP^{a1}/dDP^{a2}$ (*dDP*). (B) Quantification of cell death in *de2f1*, *de2f2*, and *dDP* single mutants. The fractional area of AO staining in each disc was quantified using Image J software. Error bars represent SEM. The data are from 7 to 15 discs per genotype per time point. At 18 h, *dDP* is statistically different from WT, *de2f1* and *de2f2* with a *p*-value of less than 0.01.

$dDP^{a1}$, together with a null allele, $dDP^{a2}$. $dDP^{a1}$ contains an amino acid substitution in the DEF box, which is required for DP/E2F heterodimerization, while $dDP^{a2}$ contains a premature stop codon in the DEF box (Royzman et al., 1997).

To our surprise, unlike *p53, de2f1* double mutants, *p53, dDP* double mutants exhibit robust apoptosis after IR, significantly more than what is seen in *p53* single mutants (Fig. 4A, quantified in B). Apoptosis in *p53, dDP* double mutants commences as early as 6 h after IR, close to the schedule of p53-dependent apoptosis. Thus, a time delay after irradiation is not a prerequisite for p53-independent apoptosis. *dDP* single mutants also exhibit robust apoptosis at times that are relevant for p53-independent apoptosis (e.g. 18 h after irradiation, Fig. 2 and data not shown). Together, these results show that dDP normally inhibits p53-independent apoptosis.

We find that *dDP* and *de2f1* mutants behave differently with regard to p53-independent apoptosis. This difference could be due to functions of dDP that are independent of dE2F1. dDP can also associate with the second E2F homolog, dE2F2. Therefore, we addressed the role of dE2F2 in p53-independent apoptosis. We find that *p53, de2f2* double mutants exhibit substantially more apoptosis 20 and 24 h after IR compared to p53 single mutant controls analyzed concurrently (Fig. 4A, quantified in C). These results indicate that dE2F2, like dDP, limits p53-independent apoptosis after IR.

dDp is a cofactor for both dE2F1 and dE2F2. The phenotype of p53 dDp double mutants suggests that IR can induce apoptosis in the absence of p53 or dE2F activity. To investigate whether this induction accompanies changes in *hid* expression, we used a *hid*>GFP reporter in which dE2F/Dp consensus sites have been mutated (Tanaka-Matakatsu et al., 2009), to be called "*hid-mut*>GFP reporter." We reasoned that the loss of these sequences would mimic that the loss of all E2F complexes (dE2F1/Dp and dE2F2/Dp) from this site. GFP expression from this reporter increased at both 4 and 24 h after exposure to 4000 R of IR in p53 heterozygous background (Fig. 5). This is consistent with the finding that in the presence of p53, apoptosis can occur with or without E2F (Fig. 2). *hid-mut*>GFP reporter did not however increase in expression after irradiation in p53 homozygous mutants (Fig. 5). There are two possible explanations for this finding. First, p53, E2F/Dp-independent apoptosis may not accompany *hid* induction. We know that robust apoptosis can take place in the absence of both p53 and E2F/Dp (Fig. 4), but we do not know the genetic basis for it. Alternatively, p53, E2F/Dp-independent apoptosis may accompany *hid* induction, but the 2 kb fragment of the *hid* promoter used here does not mediate this event. Given that the *hid* regulatory region is extensive (Grether et al., 1995), the latter possibility cannot be ruled out.

### *Mitotic index and cell death levels do not correlate*

As dE2F1 plays a key role in cell cycle regulation, we considered the possibility that the lack of apoptosis in *chk2, de2f1* and *p53, de2f1* double mutants might be an indirect result of an altered cell cycle progression. A previous work indicates that p53-independent apoptosis may be

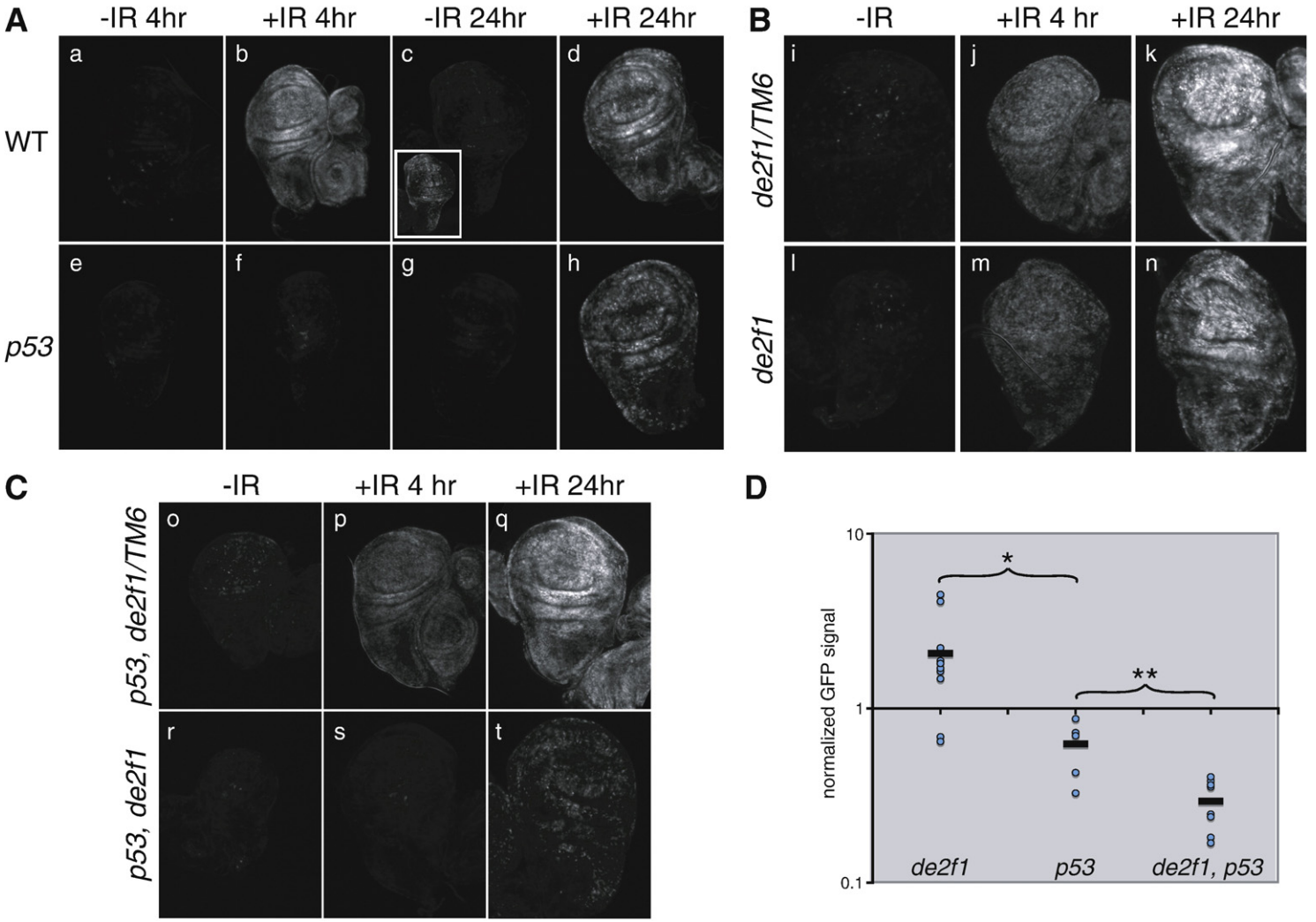


**Fig. 3.** dE2F1 is needed to activate *hid*>GFP reporter in p53 mutants after irradiation. Wing imaginal discs from the 3rd instar larvae irradiated with 0 (−IR) or 4000 R (+IR) of X-rays during the feeding stage were dissected and imaged. (A) All discs shown were imaged under identical conditions and the images were processed identically to allow for comparison. The exception is the inset in (c), which shows the disc at 4× exposure to illustrate that there is a GFP-positive disc that is not visible at the settings used for the other discs. WT = wild type; $p53 = p53^{5A\text{-}1\text{-}4}$ homozygotes. (B) The discs were imaged under identical conditions and the images were processed identically to allow for comparison. $de2f1 = de2f1^{i2}/de2f1^{7172}$;*de2f1*/TM6 = either $de2f1^{i2}$/TM6 or $de2f1^{7172}$/TM6. The balanced larvae were identified using the TB marker on TM6 balancer chromosome, and the two alleles of *de2f1* could not be differentiated. (C) The discs were imaged under identical conditions and the images processed identically to allow for comparison. *p53, de2f1* = $p53^{5A\text{-}1\text{-}4}$ $de2f1^{i2}/p53^{5A\text{-}1\text{-}4}$ $de2f1^{7172}$;*p53*· *de2f1*/TM6 = either $p53^{5A\text{-}1\text{-}4}$ $de2f1^{i2}$/TM6 or $p53^{5A\text{-}1\text{-}4}$ $de2f1^{7172}$/TM6. The balanced larvae were identified using the TB marker and the two alleles of *de2f1* could not be differentiated. (D) Mean GFP fluorescence of each disc is expressed as a fraction of average GFP fluorescence from TM6 (balancer) sibling controls (see Materials and methods for details). The average of each population is indicated with a horizontal bar. The data are from 10, 6 and 8 discs respectively from the 24 h time point in two separate experiments. One asterisk (*) denotes a statistically significant difference with a *p*-value of less than 0.05, and two asterisks (**) denote a *p*-value of less than 0.01.

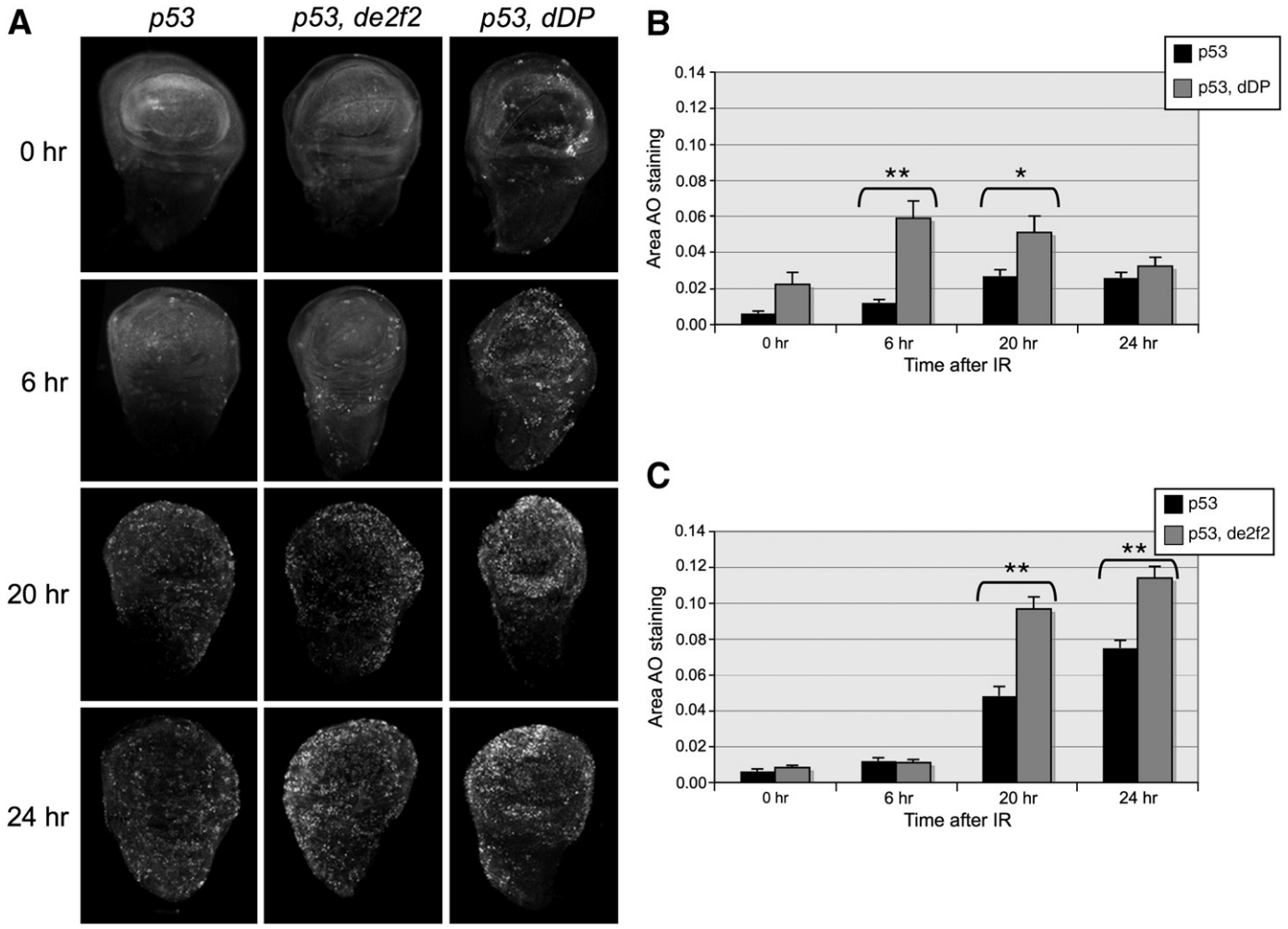


**Fig. 4.** dE2F2 and dDP limit IR-induced p53-independent apoptosis. (A) 120- to 128-hour-old (*p53* and *p53, e2f2*) or 141- to 152-hour-old (*p53, dDp*) feeding stage third-instar larvae were irradiated with 4000 R of X-rays. Imaginal discs were dissected at various times after irradiation and stained with acridine orange (AO) to detect dead cells. Genotypes are indicated for $p53^{5A\text{-}1\text{-}4}$ (p53), $p53^{5A\text{-}1\text{-}4}$;$de2f2^{329}$ (*p53, de2f2*) and $p53^{5A\text{-}1\text{-}4}$;$dDP^{a1}/dDP^{a2}$ (*p53, dDP*). (B and C) Quantification of apoptosis in *p53, dDP* and *p53, de2f2* double mutants. The fractional area of AO staining in each disc was quantified using Image J software. Error bars represent SEM. The data are from 8 to 14 discs per genotype per time point. The data are depicted separately for *p53, e2f2* (A) and *p53, dDp* (B) because of the variation in the amount of apoptosis in *p53* single mutant controls at 20 and 24 h after IR. One asterisk (*) denotes a statistically significant difference with a *p*-value of less than 0.05, and two asterisks (**) denote a *p*-value of less than 0.01.

dependent on cell cycle stage; in p53-deficient mouse epithelial cells, for example, IR-induced apoptosis occurred specifically during the G2/M phase of the cell cycle (Merritt et al., 1997). To address this possibility, we quantified mitotic cells in *chk2, de2f1* and *p53, dDP* double mutants before and after IR.

We find that wing imaginal discs from *chk2* single mutants and *chk2, de2f1* double mutants display similar numbers of mitotic cells before IR (Fig. 6). At 18 h after IR both *chk2, de2f1* double mutants and *p53, dDP* double mutants show significantly fewer mitotic cells compared to *chk2* or *p53* single mutants respectively. At this time point, *chk2, de2f1* double mutants show *less* apoptosis than *chk2* mutants whereas also *p53, dDP* double mutants show more apoptosis than *p53* mutants. Furthermore, the numbers of mitotic cells in *chk2, de2f1* and *p53, dDP* double mutants are similar, yet the level of apoptosis in these double mutants differs significantly. Since *chk2, de2f1* and *p53, dDP* double mutants exhibit similar mitotic profiles yet different levels of apoptosis, altered cell cycle progression alone cannot account for the reduction of apoptosis in *chk2, de2f1* mutants. In other words, factors other than cell cycle perturbation are needed to explain why *chk2, de2f1* mutants exhibit less apoptosis than *chk2* single mutants and why *p53, dDP* mutants exhibit more apoptosis than *p53* single mutants.

### *Chk2-/p53-independent apoptosis is important for survival and development after IR*

Chk2-/p53-dependent apoptosis is deemed to be important for culling damaged cells and preserving genetic integrity after radiation exposure (Brodsky et al., 2000b; Sogame et al., 2003). p53-independent apoptosis was recently shown to reduce genetic instability in assays for loss of heterozygosity (LOH) (McNamee and Brodsky, 2009). Here, we addressed whether p53-independent apoptosis has a role in the proper development and survival of organisms. We reasoned that LOH would be more detrimental if the organism is able to survive and pass it onto the next generation.

Reducing *hid* gene dosage in *chk2* mutants compromises Chk2-/p53-independent apoptosis in wing imaginal discs (Figs. 7A and B). Adult wings that result from such mutants show defects in an IR-dependent manner; 45% of wings show defects in *chk2/chk2; hid/TM6* (CyO and TM6 are balancer chromosomes). The reduction of *hid* dosage alone did not have as severe an effect (9% in *chk2/CyO; hid/TM6*). *chk2* homozygosity alone also did not have as severe an effect; 16% of wings from 87 adults of *chk2/chk2; Sb/TM6* that were irradiated as the 3rd instar larvae show defects (Sb is a visible marker on Chromosome III and serves as a control). The difference between

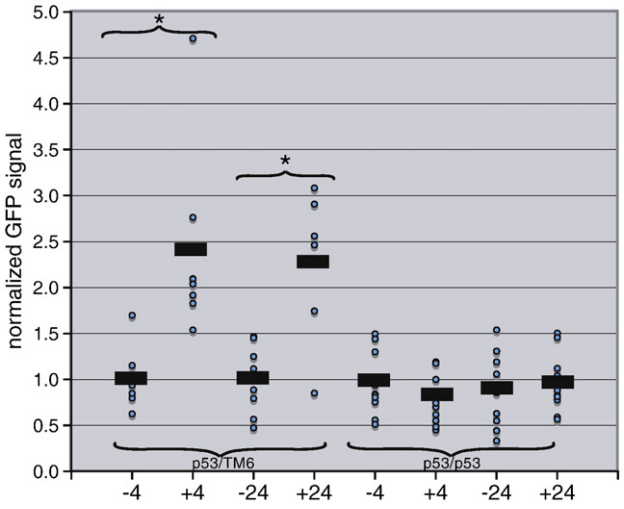


**Fig. 5.** Mutations in E2F consensus sites alter the response of *hid>GFP* to radiation and p53 status. The 3rd instar larvae were irradiated with 0 (−) or 4000R (+) of X-rays during the feeding stage. Wing imaginal discs were dissected at either 4 or 24 h after irradiation and imaged. All were homozygotes for the *hid-mut*>GFP transgene but were either homozygous for $p53^{5A\text{-}1\text{-}1}$ or heterozygous with a balancer chromosome (TM6). The mean GFP fluorescence of each disc is expressed as a fraction of average GFP fluorescence from un-irradiated TM6 (balanced) sibling controls (see Materials and methods for details). The average of each population is indicated with a horizontal bar. The data are from 6 to 11 discs per sample in two separate experiments. One asterisk (*) denotes a statistically significant difference with a *p*-value of less than 0.01.

*chk2/chk2; hid/TM6* and either of the controls is statistically significant ($p<0.001$). The detrimental effect of blocking chk2-/p53-independent apoptosis was confirmed in the p53 mutant background (Fig. S1). We used GMR to express caspase inhibitor p35 in eye imaginal discs of *p53* mutants and investigated the effect on eye development. In *p53* mutants, where p53-independent apoptosis would occur after irradiation, the resulting adult eyes show little defect. Blocking p53-independent apoptosis with GMR>p35, in contrast, produced eye deformities in an IR-dependent manner.

Finally, following irradiation, the number of chk2 homozygous adults (*chk2/chk2; hid/TM6*) recovered relative to heterozygotes (*chk2/CyO; hid/TM6*) was less than the expected Mendelian ratio (CyO and TM6 are balancer chromosomes). Without irradiation, 180 Cy to 92 Cy+ adults were recovered, which fits with the expected 2:1 ratio ($0.8>p>0.9$ by $\chi^2$ test). Irradiation of larvae with 3000 R of X-rays produced 153 Cy to 47 Cy+ adults, which deviates significantly from the 2:1 ratio ($0.0.001>p>0.01$ by $\chi^2$ test). We conclude that the ability to kill cells in a p53-independent (Fig. S1) or Chk2-independent (Fig. 7) manner is important for proper development and organism survival after irradiation.

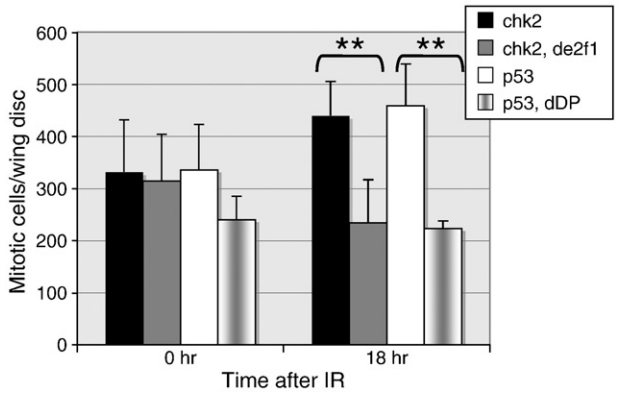


**Fig. 6.** Mitotic index is similar in *chk2, de2f1* and *p53, dDP* double mutants. 118- to 126-hour-old feeding stage third-instar larvae were irradiated with 4000 R of X-rays. Wing discs were dissected at 0 and 18 h after IR, fixed and stained with an antibody to phospho-histone H3 to detect mitotic cells. The numbers of mitotic cells per wing disc were quantified and averaged for 6–9 discs per genotype per time point. Asterisks (**) denote a statistically significant difference with a *p*-value of less than 0.01. Genotypes are indicated for $mnk^{p6}$ (*chk2*), $mnk^{p6}$;$de2f1^{i2}/de2f1^{7172}$ (*chk2, de2f1*), $p53^{5A\text{-}1\text{-}4}$ (*p53*) and $p53^{5A\text{-}1\text{-}4}$;$dDP^{a1}/dDP^{a2}$ (*p53, dDP*).

## Discussion

We have taken advantage of the relative simplicity of *Drosophila* E2F and p53 families to study the role of E2Fs in p53-independent apoptosis. Our results indicate that *Drosophila* E2F homologs play opposing roles in regulating p53-independent apoptosis in response to IR. We find that dE2F1, a homolog of the mammalian "activator" E2Fs, is required for Chk2-/p53-independent apoptosis, while dE2F2, a homolog of the mammalian "repressor" E2Fs, limits p53-independent apoptosis. The net E2F activity in the cell, reduced by mutations in dDP, is inhibitory towards p53-independent apoptosis.

One surprising finding from these studies is that 2 kb of *hid* promoter confers IR-induced transcriptional activation in a p53-dependent manner (Fig. 3). This is surprising because in embryos, transcriptional activation of *hid* by IR in a p53-dependent manner requires the IRER (irradiation responsive enhancer region) that lies next to *rpr*, ~200 kb away from *hid*, and is regulated epigenetically by histone modification (Zhang et al., 2008). Yet, as shown previously, 2 kb of *hid* promoter is enough to allow IR-induced GFP expression in eye and wing imaginal discs (Tanaka-Matakatsu et al., 2009). Here we show that this induction is p53-dependent. Clearly, regulation of *hid* by IR is different between embryos and larval discs.

Mammalian p53 consensus is a tandem repeat of 10 nucleotides with the sequence RRR**C**WW**G**YYY where R = G/A, W = A/T and Y = T/C and invariant C and G are shown in bold (Brodsky et al., 2000a). *Drosophila* p53 binds to a DNA damage response element at the *rpr* locus that differs from the mammalian consensus at one position shown in lower case; tGA**C**ATT**G**TTT GAA**C**AA**G**TCg (Brodsky et al., 2000a). Manual examination of 2 kb of *hid* promoter fragment that responds to p53 status shows a potential binding sequence at −2006 from the start of *hid* transcription that deviates from the mammalian consensus at two positions, and another at −1667 that deviates at three positions. These are ttG**C**AT**G**CTC Gct**C**AT**G**TTC and GtG**C**AA**G**agT GtG**C**TT**G**aat respectively. Since the consensus for *Drosophila* p53 has not been determined, it is possible that either or both of these are responsible for the effect of p53 on *hid*-GFP reporter.

The 2 kb *hid* enhancer includes E2F consensus sequences. Rb has been shown to repress the expression of *hid*>GFP reporter when E2F binding sequences are intact but not when these are mutated (Tanaka-Matakatsu et al., 2009). That is, E2F binding sites allow for repression of *hid* by Rb although which E2F mediates this repression is not known. In any case, our finding that net E2F activity is inhibitory towards apoptosis would be consistent with the published result that Rb inhibits *hid* expression via E2F consensus sites. We do not know if dE2F1 plays a permissive role (e.g. by allowing elevated basal expression of pro-apoptotic genes) or an instructive role (e.g. by allowing for induction of pro-apoptotic genes by IR), or both. The results with *hid*>GFP reporter (e.g. Fig. 3C) are consistent with an instructive role but do not rule out a permissive role.

In the absence of p53, dE2F1 is needed for the transcriptional induction of *hid*>GFP reporter by IR (Fig. 3C). This can explain two published results: that *hid* is necessary for IR-induced p53-independent apoptosis (McNamee and Brodsky, 2009), and that *hid* is transcriptionally induced in p53 mutants after a time delay (Wichmann et al., 2006). Human E2F1 can bind to the promoter of a *hid* ortholog, *Smac*/DIABLO, and can, when ectopically expressed, activate the transcription of the latter *in vivo* (Xie et al., 2006). The role of p53 status in this process or the significance of this mode of

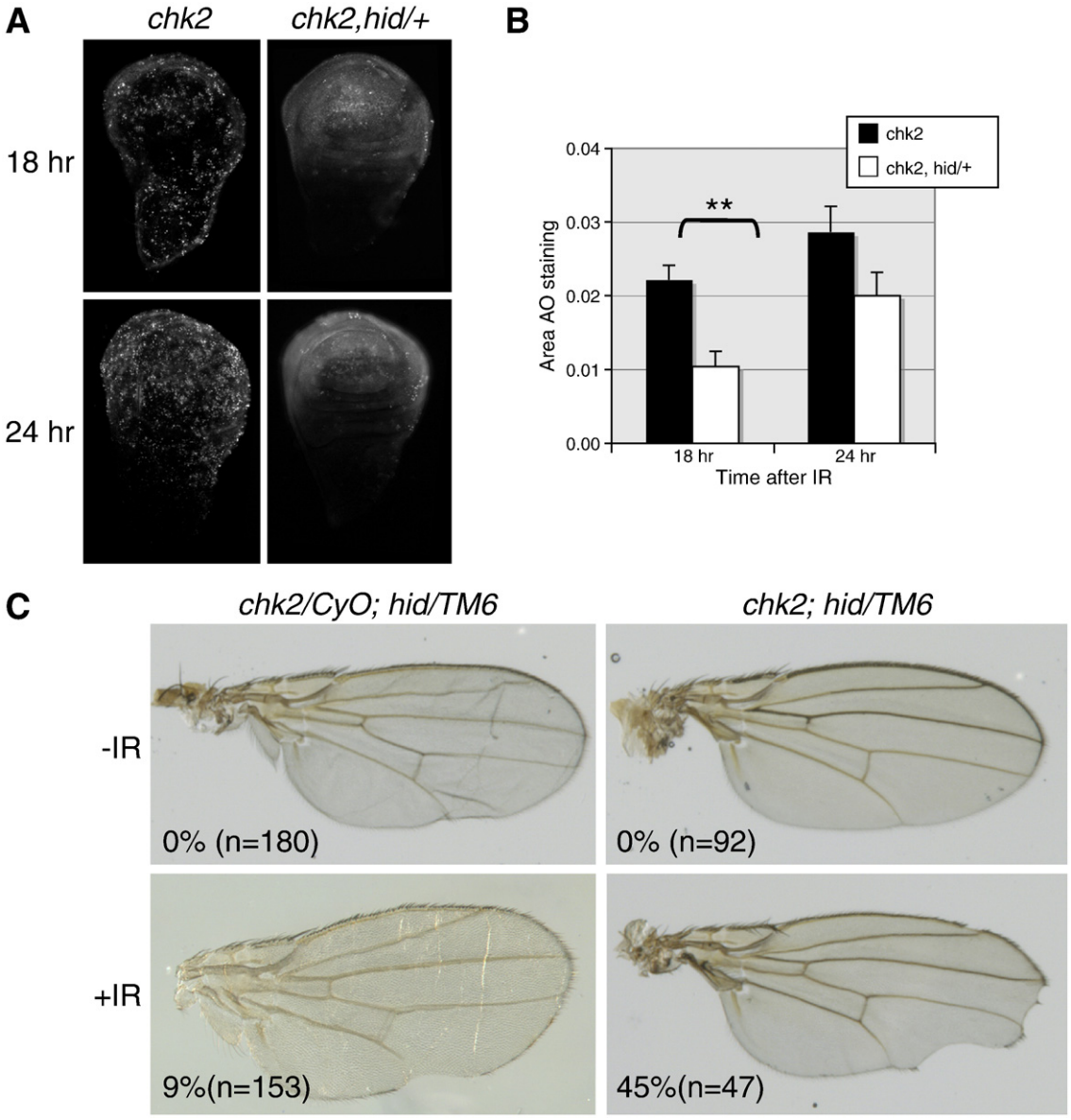


**Fig. 7.** The consequences of blocking Chk2-independent apoptosis. (A) 118- to 128-hour-old feeding stage third-instar larvae were irradiated with 4000 R of X-rays. Imaginal discs were dissected at various times after irradiation and stained with acridine orange (AO) to detect dead cells. Genotypes are indicated for $mnk^{p6}$ (*chk2*) and $mnk^{p6}$;$hid^{05014}$/*TM6-Tb* (*chk2, hid*/+). (B) The fractional area of AO staining in each disc was quantified using Image J software. The data are from 19 to 21 discs per genotype per time point. Asterisks (**) denote a statistically significant difference with a *p*-value of less than 0.01. (C) Wing defects in adults in three independent experiments are quantified. Defective wings show missing parts as in the examples in the bottom panels. Data shown are from larvae that have been irradiated with 0 (−IR) or 3000 (+IR) R of X-rays. The percent of adults with wing defects that result from irradiation is significantly different between the two genotypes shown ($p<0.001$, Fisher's exact test). Note that wings from CyO animals had to be un-curled before imaging, which can introduce wrinkles (e.g. wings in two left panels). CyO and TM6 are balancer chromosomes.

regulation was not investigated. We speculate that the role of E2F1 in IR-induced, p53-independent transcriptional activation of *Smac*/DIABLO genes may be conserved in mammals.

Previous work has shown that dE2F1 and dE2F2 exhibit antagonistic functions, with dE2F1 activating and dE2F2 repressing the transcription of a reporter containing canonical E2F sites from the PCNA promoter. dE2F1 and dE2F2 occupy the PCNA promoter and the ratio of the two E2F proteins influenced the degree of transcriptional activation or repression (Frolov et al., 2001). In wild type, PCNA expression is tightly coupled to the pattern of S phase, in eye imaginal discs for example. In *de2f2* mutants, PCNA is no longer down-regulated outside pattern of the S phase. The loss of all E2F activities, either in *de2f1, de2f2* double mutants or in *dDP* single mutants, results in de-repression of PCNA such that a low but significant level is expressed throughout the cell cycle. Thus the net result of opposing E2F activities is the cyclical expression of PCNA in concert with DNA replication.

The paradigm of E2F-dependent regulation of PCNA helps us understand the role of E2F proteins in p53-independent apoptosis. dE2F1 and dE2F2 might similarly influence p53-independent apoptosis by regulating pro-apoptotic gene(s) such as *hid*. According to this model, dE2F2 (with dDp) provides a net repressive activity that inhibits IR-induced apoptosis. This activity must be operative only in the absence of p53;dE2F2 mutations that have no effect on apoptosis when p53 is present (Moon et al., 2005). In p53 mutants, dE2F1 counteracts dE2F2 after irradiation and thus promotes apoptosis. Disabling transcriptional activation by dE2F1, which is what the allelic combination $de2f1^{i2}/de2f1^{7172}$ is predicted to cause, would result in the failure to overcome dE2F2/Dp. Removal of dE2F2 with null alleles, would result in increased gene expression and more apoptosis.

Reducing the ability of dDP to interact with dE2Fs, which is what the allelic combination $dDP^{a1}/dDP^{a2}$ is predicted to cause, would reduce dE2F1 and dE2F2 activities simultaneously. Since this results in more apoptosis, the net E2F activity is inhibitory on apoptosis when p53 is absent.

This study and published studies in wing imaginal discs (e.g. Moon et al., 2005) reveal significant differences in the effect of E2F/DP mutations on p53-dependent apoptosis (typically assayed at 4–6 h post irradiation) and p53-independent apoptosis (18–24 h after irradiation in p53 mutants). The clearest difference is that dE2F2 null mutations have little or no effect on p53-dependent apoptosis (Moon et al., 2005), but increase the level of p53-independent apoptosis (Fig. 4). dDP loss-of-function mutations decrease p53-dependent apoptosis throughout eye imaginal discs and in most cells of the wing pouch (Moon et al., 2005, 2008), whereas they increase the level of p53-independent apoptosis (Fig. 4). In contrast, loss-of-function mutations in dE2F1 reduced both p53-dependent apoptosis in most cells of the wing imaginal disc (Moon et al., 2005) and p53-independent apoptosis in the wing imaginal disc (Fig. 1). These differences raise the question 'how does p53 status alter the role of dE2F2 and dDP in IR-induced apoptosis?' In the presence of p53, dE2F2 has little effect and dDP is stimulatory. In the absence of p53, dE2F2 and dDP play inhibitory roles. Perhaps the occupancy of transcriptional factors at the target loci such as the *hid* promoter is sensitive to p53 status.

In the eye imaginal disc, mutations in *ago*, a ubiquitin ligase, result in elevated apoptosis (Nicholson et al., 2009). This mode of cell death occurs via elevated E2F1 activity, increased expression of *hid* and *rpr* and is independent of apoptosis. Thus, the role of dE2F1 in promoting p53-independent apoptosis is conserved in another tissue of the larvae.

## Conclusions

Previous studies found that the role of *Drosophila* E2F transcription factors in apoptosis is context-dependent and is influenced by, for example, whether the cells are in the wing pouch or at the dorsal/ventral margin of the wing disc and whether apoptosis is induced by radiation or by the loss of a tumor suppressor homolog, Rb (Moon et al., 2005, 2008; Tanaka-Matakatsu et al., 2009). The current study addresses the role of E2F family members in IR-induced p53-independent apoptosis. The most significant finding here is that reducing E2F activity, as in the case of dDP mutants, allows p53-null cells to die following IR exposure. This is in clear contrast to the finding that a similar reduction of E2F activity prevents p53 wild type cells from dying following IR exposure (Moon et al., 2005). Several E2F antagonists are being considered in cancer therapy (La Thangue and Bandara, 2002). Our results from *Drosophila* studies would caution that p53 status must be considered when using such therapies in conjunction with radiation treatment. If the findings in *Drosophila* apply to human cancers, an E2F antagonist would help kill p53-deficient cancer cells following radiation treatment, but would help p53-wild type cancer cells survive. In addition, an E2F antagonist may be particularly suitable for combination therapy with radiation to eradicate p53-deficient tumors because it may sensitize p53-deficient cancer cells to radiation while protecting p53-wild type somatic cells from the cell-killing effects of IR.

## Materials and methods

### *Fly stocks*

WT flies were of the Sevelin stock. All fly mutants used here have been described before. $mnk^{p6}$ (*chk2*) (Brodsky et al., 2004) and $p53^{5A\text{-}1\text{-}4}$ (Rong et al., 2002) are null alleles that result from partial deletion of each gene. $de2f1^{i2}$ is a hypomorphic allele that contains a premature stop codon that is predicted to produce a truncated protein that contains the DNA binding domain, but lacks the Rb-binding and transcriptional activation region (Royzman et al., 1999). $de2f1^{7172}$ is a null allele created by a P-element insertion 33 nucleotides upstream of the start site (Duronio et al., 1995). $dDP^{a1}$ is a hypomorphic allele due to R149C mutation in the DEF box, and $dDP^{a2}$ is a null allele due to a W173STOP mutation (Royzman et al., 1997). $de2f2^{329}$ is a null allele due to a partial deletion of the gene (Cayirlioglu et al., 2001). $de2f2^{76Q}$ is a null allele due to a deletion from −378 bp to the 5th codon of the ORF (Frolov et al., 2001). $hid^{05014}$ contains a P-element insertion between amino acids 105 and 106 (Grether et al., 1995). GMR-p35 is an insertion on the X chromosome (Hay et al., 1994). *hid*>GFP and *hid-mut*>GFP are insertions on chromosome II (Tanaka-Matakatsu et al., 2009).

### *Irradiation*

Feeding 3rd instar larvae in food were irradiated using a TORREX X-ray generator, set at 115 kV and 5 mA (producing 2.4 rad/s).

### *Acridine orange staining*

Larvae were dissected in PBS. Imaginal discs were incubated for 2.5 min in PBS + 0.5 mM acridine orange (Sigma) at room temperature, washed once with PBS, mounted in PBS, and imaged immediately using a Leica DMR fluorescence compound microscope, a Sensicam CCD camera and Slidebook software (Intelligent Imaging, Inc.). Acridine orange signal was quantified using the Image J software from NIH (http://rsb.info.nih.gov/ij/).

### *Antibody staining and microscopy*

To detect phosphorylated histone H3, larval imaginal discs were extirpated in PBS, fixed for 20 min in PBTx (PBS with 0.3% Triton-X) containing 5% formaldehyde and washed 3 times with PBTx. Samples were incubated 2 h at room temperature (25 °C) or overnight at 4 °C with a polyclonal anti-phospho histone H3 antibody (pH3; Upstate Biotechnology) diluted to 1:1000 in blocking solution, which is PBTx + 10% Normal Goat Serum. Following incubation with the pH3 antibody, samples were washed 3 times with PBTx and incubated 2–4 h at room temperature with anti-rabbit secondary antibody conjugated to Rhodamine, diluted to 1:500 in blocking solution (Jackson ImmunoResearch). Samples were washed 3 times with PBTx, stained with 10 mg/ml Hoechst33258 in PBTx for 2 min, and washed 3 times with PBTx before mounting onto slides with Fluoromount G. Samples were imaged on a Leica DMR fluorescence microscope using a Sensicam CCD camera and Slidebook software (Intelligent Imaging, Inc.). Mitotic cells were counted manually. Images of adult eyes and wings were acquired using an Olympus dissecting microscope, a CCD camera and SPOT software (Diagnostic Instruments), and processed using Photoshop.

### *Quantification of GFP*

The discs were stained with Hoechst33432 (live cell permeable DNA dye; Molecular Probes) and imaged for GFP and for Hoechst. The images were collected using a Leica DMR fluorescence compound microscope, 10× objective, a Sensicam CCD camera and Slidebook software (Intelligent Imaging, Inc.). Images were converted to TIFF. Mean GFP and mean DNA fluorescence signals for each disc were quantified using Image J software from NIH (http://rsb.info.nih.gov/ij/). The GFP signal was then normalized to the DNA signal for each disc. This, we found, helped correct for different extent of flattening of the disc. Normalized GFP signal was averaged for all discs from TM6 balancer controls for each genotype. Normalized GFP signal for each experimental disc was

then expressed as a fraction of average TM6 GFP from the same experiment.

*Statistical analysis*

Fisher's exact test was used to analyze adult phenotypes in Fig. 6 and Fig. S1. $\chi^2$ test was used to determine the significance of radiation survival data. Student's *t*-test was used to calculate statistical significance of all other data sets.

## Acknowledgments

We thank Heinrich Jasper, Bruce Hays, Michael Brodsky, Wei Du, Bob Duronio, Terry Orr-Weaver and the Bloomington Stock Center for fly stocks, and Carina Kee for technical assistance with image analysis using Image J. This work was initiated with RO1 GM66441 and completed with RO1 GM87276, both to T. T. Su.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.07.023.

## References

Abrams, J.M., White, K., Fessler, L.I., Steller, H., 1993. Programmed cell death during Drosophila embryogenesis. Development 117, 29–43.
Asano, M., Nevins, J.R., Wharton, R.P., 1996. Ectopic E2F expression induces S phase and apoptosis in *Drosophila* imaginal discs. Genes Dev. 10, 1422–1432.
Bandara, L.R., Buck, V.M., Zamanian, M., Johnston, L.H., La Thangue, N.B., 1993. Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F. EMBO J. 12, 4317–4324.
Beijersbergen, R.L., Kerkhoven, R.M., Zhu, L., Carlee, L., Voorhoeve, P.M., Bernards, R., 1994. E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. Genes Dev. 8, 2680–2690.
Brodsky, M.H., Nordstrom, W., Tsang, G., Kwan, E., Rubin, G.M., Abrams, J.M., 2000a. *Drosophila* p53 binds a damage response element at the reaper locus. Cell 101, 103–113.
Brodsky, M.H., Sekelsky, J.J., Tsang, G., Hawley, R.S., Rubin, G.M., 2000b. mus304 encodes a novel DNA damage checkpoint protein required during *Drosophila* development. Genes Dev. 14, 666–678.
Brodsky, M.H., Weinert, B.T., Tsang, G., Rong, Y.S., McGinnis, N.M., Golic, K.G., Rio, D.C., Rubin, G.M., 2004. *Drosophila melanogaster* MNK/Chk2 and p53 regulate multiple DNA repair and apoptotic pathways following DNA damage. Mol. Cell. Biol. 24, 1219–1231.
Cayirlioglu, P., Bonnette, P.C., Dickson, M.R., Duronio, R.J., 2001. *Drosophila* E2f2 promotes the conversion from genomic DNA replication to gene amplification in ovarian follicle cells. Development 128, 5085–5098.
Cuddihy, A.R., Bristow, R.G., 2004. The p53 protein family and radiation sensitivity: yes or no? Cancer Metastasis Rev. 23, 237–257.
DeGregori, J., 2002. The genetics of the E2F family of transcription factors: shared functions and unique roles. Biochim. Biophys. Acta 1602, 131–150.
DeGregori, J., Johnson, D.G., 2006. Distinct and overlapping roles for E2F family members in transcription, proliferation and apoptosis. Curr. Mol. Med. 6, 739–748.
Du, W., Xie, J.E., Dyson, N., 1996. Ectopic expression of dE2F and dDP induces cell proliferation and death in the *Drosophila* eye. EMBO J. 15, 3684–3692.
Duronio, R.J., Bonnette, P.C., O'Farrell, P.H., 1998. Mutations of the *Drosophila* dDP, dE2F, and cyclin E genes reveal distinct roles for the E2F-DP transcription factor and cyclin E during the G1-S transition. Mol. Cell. Biol. 18, 141–151.
Duronio, R.J., O'Farrell, P.H., Xie, J.E., Brook, A., Dyson, N., 1995. The transcription factor E2F is required for S phase during *Drosophila* embryogenesis. Genes Dev. 9, 1445–1455.
Dynlacht, B.D., Brook, A., Dembski, M., Yenush, L., Dyson, N., 1994. DNA-binding and trans-activation properties of *Drosophila* E2F and DP proteins. Proc. Natl Acad. Sci. USA 91, 6359–6363.
Frolov, M.V., Huen, D.S., Stevaux, O., Dimova, D., Balczarek-Strang, K., Elsdon, M., Dyson, N.J., 2001. Functional antagonism between E2F family members. Genes Dev. 15, 2146–2160.
Grether, M.E., Abrams, J.M., Agapite, J., White, K., Steller, H., 1995. The head involution defective gene of *Drosophila melanogaster* functions in programmed cell death. Genes Dev. 9, 1694–1708.
Hay, B.A., Wolff, T., Rubin, G.M., 1994. Expression of baculovirus P35 prevents cell death in *Drosophila*. Development 120, 2121–2129.
Holmberg, C., Helin, K., Sehested, M., Karlstrom, O., 1998. E2F-1-induced p53-independent apoptosis in transgenic mice. Oncogene 17, 143–155.
Irwin, M., Marin, M.C., Phillips, A.C., Seelan, R.S., Smith, D.I., Liu, W., Flores, E.R., Tsai, K.Y., Jacks, T., Vousden, K.H., Kaelin Jr., W.G., 2000. Role for the p53 homologue p73 in E2F-1-induced apoptosis. Nature 407, 645–648.
La Thangue, N.B., Bandara, L.R., 2002. Targets for cancer chemotherapy: transcription factors and other nuclear proteins. Humana Press, Totowa, NJ.
Ma, Y., Croxton, R., Moorer Jr., R.L., Cress, W.D., 2002. Identification of novel E2F1-regulated genes by microarray. Arch. Biochem. Biophys. 399, 212–224.
McNamee, L.M., Brodsky, M.H., 2009. p53-independent apoptosis limits DNA damage-induced aneuploidy. Genetics 182, 423–435.
Merritt, A.J., Allen, T.D., Potten, C.S., Hickman, J.A., 1997. Apoptosis in small intestinal epithelial from p53-null mice: evidence for a delayed, p53-independent G2/M-associated cell death after gamma-irradiation. Oncogene 14, 2759–2766.
Milton, A., Luoto, K., Ingram, L., Munro, S., Logan, N., Graham, A.L., Brummelkamp, T.R., Hijmans, E.M., Bernards, R., La Thangue, N.B., 2006. A functionally distinct member of the DP family of E2F subunits. Oncogene 25, 3212–3218.
Moon, N.S., Di Stefano, L., Morris, E.J., Patel, R., White, K., Dyson, N.J., 2008. E2F and p53 induce apoptosis independently during *Drosophila* development but intersect in the context of DNA damage. PLoS Genet. 4, e1000153.
Moon, N.S., Frolov, M.V., Kwon, E.J., Di Stefano, L., Dimova, D.K., Morris, E.J., Taylor-Harding, B., White, K., Dyson, N.J., 2005. *Drosophila* E2F1 has context-specific pro- and antiapoptotic properties during development. Dev. Cell 9, 463–475.
Moroni, M.C., Hickman, E.S., Lazzerini Denchi, E., Caprara, G., Colli, E., Cecconi, F., Muller, H., Helin, K., 2001. Apaf-1 is a transcriptional target for E2F and p53. Nat. Cell Biol. 3, 552–558.
Morris, L., Allen, K.E., La Thangue, N.B., 2000. Regulation of E2F transcription by cyclin E-Cdk2 kinase mediated through p300/CBP co-activators. Nat. Cell Biol. 2, 232–239.
Nahle, Z., Polakoff, J., Davuluri, R.V., McCurrach, M.E., Jacobson, M.D., Narita, M., Zhang, M.Q., Lazebnik, Y., Bar-Sagi, D., Lowe, S.W., 2002. Direct coupling of the cell cycle and cell death machinery by E2F. Nat. Cell Biol. 4, 859–864.
Nicholson, S.C., Gilbert, M.M., Nicolay, B.N., Frolov, M.V., Moberg, K.H., 2009. The archipelago tumor suppressor gene limits rb/e2f-regulated apoptosis in developing *Drosophila* tissues. Curr. Biol. 19, 1503–1510.
Ohtani, K., Nevins, J.R., 1994. Functional properties of a *Drosophila* homolog of the E2F1 gene. Mol. Cell. Biol. 14, 1603–1612.
Ollmann, M., Young, L.M., Di Como, C.J., Karim, F., Belvin, M., Robertson, S., Whittaker, K., Demsky, M., Fisher, W.W., Buchman, A., Duyk, G., Friedman, L., Prives, C., Kopczynski, C., 2000. *Drosophila* p53 is a structural and functional homolog of the tumor suppressor p53. Cell 101, 91–101.
Rogers, K.T., Higgins, P.D., Milla, M.M., Phillips, R.S., Horowitz, J.M., 1996. DP-2, a heterodimeric partner of E2F: identification and characterization of DP-2 proteins expressed in vivo. Proc. Natl Acad. Sci. USA 93, 7594–7599.
Rong, Y.S., Titen, S.W., Xie, H.B., Golic, M.M., Bastiani, M., Bandyopadhyay, P., Olivera, B.M., Brodsky, M., Rubin, G.M., Golic, K.G., 2002. Targeted mutagenesis by homologous recombination in *D. melanogaster*. Genes Dev. 16, 1568–1581.
Royzman, I., Austin, R.J., Bosco, G., Bell, S.P., Orr-Weaver, T.L., 1999. ORC localization in *Drosophila* follicle cells and the effects of mutations in dE2F and dDP. Genes Dev. 13, 827–840.
Royzman, I., Whittaker, A.J., Orr-Weaver, T.L., 1997. Mutations in *Drosophila* DP and E2F distinguish G1-S progression from an associated transcriptional program. Genes Dev. 11, 1999–2011.
Sawado, T., Yamaguchi, M., Nishimoto, Y., Ohno, K., Sakaguchi, K., Matsukage, A., 1998. dE2F2, a novel E2F-family transcription factor in *Drosophila melanogaster*. Biochem. Biophys. Res. Commun. 251, 409–415.
Shu, H.K., Julin, C.M., Furman, F., Yount, G.L., Haas-Kogan, D., Israel, M.A., 2000. Overexpression of E2F1 in glioma-derived cell lines induces a p53-independent apoptosis that is further enhanced by ionizing radiation. Neuro-oncology 2, 16–21.
Sogame, N., Kim, M., Abrams, J.M., 2003. Drosophila p53 preserves genomic stability by regulating cell death. Proc. Natl Acad. Sci. USA 100, 4696–4701.
Tanaka-Matakatsu, M., Xu, J., Cheng, L., Du, W., 2009. Regulation of apoptosis of rbf mutant cells during *Drosophila* development. Dev. Biol. 326, 347–356.
Wichmann, A., Jaklevic, B., Su, T.T., 2006. Ionizing radiation induces caspase-dependent but Chk2- and p53-independent cell death in *Drosophila melanogaster*. PNAS 103, 9952–9957.
Wu, C.L., Zukerberg, L.R., Ngwu, C., Harlow, E., Lees, J.A., 1995. In vivo association of E2F and DP family proteins. Mol. Cell. Biol. 15, 2536–2546.
Xie, W., Jiang, P., Miao, L., Zhao, Y., Zhimin, Z., Qing, L., Zhu, W.G., Wu, M., 2006. Novel link between E2F1 and Smac/DIABLO: proapoptotic Smac/DIABLO is transcriptionally upregulated by E2F1. Nucleic Acids Res. 34, 2046–2055.
Zhang, Y., Chellappan, S.P., 1995. Cloning and characterization of human DP2, a novel dimerization partner of E2F. Oncogene 10, 2085–2093.
Zhang, Y., Lin, N., Carroll, P.M., Chan, G., Guan, B., Xiao, H., Yao, B., Wu, S.S., Zhou, L., 2008. Epigenetic blocking of an enhancer region controls irradiation-induced proapoptotic gene expression in *Drosophila* embryos. Dev. Cell 14, 481–493.
Zhou, L., Steller, H., 2003. Distinct pathways mediate UV-induced apoptosis in *Drosophila* embryos. Dev. Cell 4, 599–605.